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Dithiothreitol increases β-glucuronidase accumulation in transformed tobacco (*Nicotiana tabacum*) protoplasts without altering their viability or the synthesis and export of cellular proteins

Abstract The effect of dithiothreitol (DTT) on the expression of the β-glucuronidase (GUS) reporter gene under the control of the CaMV-35 S promoter has been investigated by radioactive labelling and immunoprecipitation of the enzyme in protoplasts from stably transformed tobacco plants and compared with that observed in protoplasts transiently expressing the same gene construct. An increase in net accumulation of GUS during the culture period in response to externally added DTT (2 mM) was observed both in protoplasts from transformed tobacco plants and in electroporated protoplasts. DTT had no effect on rate of degradation of the mature GUS protein, as shown in a pulse-chase experiment. Relevant aspects of protoplast physiology, such as viability, synthesis of ^{35}S-labelled cellular proteins, or synthesis and export of pathogenesis-related proteins (one putative chitinase and two 1,3-β-glucanases) were not affected by the reducing reagent.

Key words Dithiothreitol · β-Glucuronidase · Redox modulation · Tobacco protoplasts

Abbreviations *DTT* Dithiothreitol · *ER* Endoplasmic reticulum · *GUS* β-Glucuronidase · *PR* Pathogenesis related

Introduction

Recent experiments performed with animal cells indicate that externally added thiol reagents interfere with various processes affecting proteins that are translocated into the endoplasmic reticulum (ER), including assembly and secretion (Alberini et al. 1990), folding and oligomerization (Braakman et al. 1992a, b; Tatu et al. 1993) and, in some cases, sorting among different pathways (Chanat et al. 1993). We have previously found that transient expression in plant protoplasts of reporter genes encoding cysteine-rich bacterial enzymes, such as β-glucuronidase (GUS) and neomycin phosphotransferase II, is increased by one order of magnitude by externally added dithiothreitol (DTT), irrespective of whether the proteins are directed to the cytosol or to the ER (Piñeiro et al. 1994). The increase in GUS activity was small when protoplasts from transformed plants were exposed to DTT under the same conditions. Although it was speculated that protein accumulated in these protoplasts prior to treatment with the reducing agent was obscuring the effect on protein accumulation during the treatment period, it could not be excluded that the additional stress produced by the transient expression procedures (Jones et al. 1987; Díaz 1994) was making the protoplasts more sensitive to DTT. DTT is a non-physiological, membrane-permeant reducing agent that is able to prevent disulfide bond formation in newly synthesized proteins and to induce reduction of partially oxidized folding intermediates in the ER, while not significantly affecting translation, translocation, glycosylation, signal sequence removal, or function of the secretory pathway and the Golgi complex (Braakman et al. 1992a, b; Tatu et al. 1993).

We now show that DTT-mediated enhancement of GUS expression in protoplasts from transformed tobacco is similar to that produced during transient expression, and that the enhancement is not due to a differential effect of DTT on the degradation rate of mature GUS. We also show that DTT does not affect relevant aspects of protoplast physiology, such as viability, synthesis of sulfur-containing cellular proteins, or synthesis and export of pathogenesis-related (PR) proteins.

Materials and methods

Protoplast isolation and culture, electroporation, and enzyme activity

A gene fusion involving the coding sequence for GUS and the CaMV-35S promoter (35S-*gus*), was from our previous work (Díaz et al. 1992). Leaf protoplasts were isolated from axenic shoot cultures of transformed (35S-*gus*) and non-transformed *Nicotiana tabacum* W38 following established procedures (Power et al. 1989). Purified protoplasts were incubated at a concentration of $2.5–5\times10^5$/ml in MSP_19 M medium for 20 h at 25 °C in the dark, in the presence of the indicated concentrations of DTT. Protoplasts were transiently transfected by electroporation at 48 Ω, 500 µF, 750 V/cm, 20–30 ms (Piñeiro et al. 1994), using an Electro Cell Manipulator 600 (BTX electroporation system). Protoplast viability was determined by staining with Evan's blue (Gaff and Okong'O-ogola, 1971). GUS activity in protoplasts was measured by fluorometric assay (Jefferson 1987). Protein was determined with the Bradford reagent (BioRad), using bovine serum albumin as standard.

Western blotting, immunoprecipitation, and autoradiography

Cellular extracts and culture media were brought to 70% saturation with ammonium sulfate in the presence of 0.3 mg/ml BSA. Precipitated proteins were separated by SDS-PAGE on 4–20% polyacrylamide minigels (Bio-Rad). Proteins were blotted onto Immobilon P membranes (Millipore) which were then incubated with antibodies against chitinase P-32 and glucanase Glu-36 following established procedures. The antibodies, which were the kind gift of Prof. V. Conejero (Valencia, Spain), had been raised against chitinase P-32 and 1,3-β-glucanase Glu-36 from tomato and recognize one and two putative homologues produced by tobacco protoplasts, respectively (Legrand et al. 1987; Kauffmann et al. 1987).

Total protein was labelled with TRANS35 S label (ICN) by incubating the protoplasts for 8 h in MSP_19 M at 25 °C in the presence of the labelled amino acid mix. Immunoprecipitation with anti-GUS antibodies (5 Prime-3 Prime) was carried out according to the supplier's instructions. After SDS-PAGE (Laemmli 1970) of total or immunoprecipitated protein, the gels were incubated in fluorographic reagent (Amersham) and dried for autoradiography.

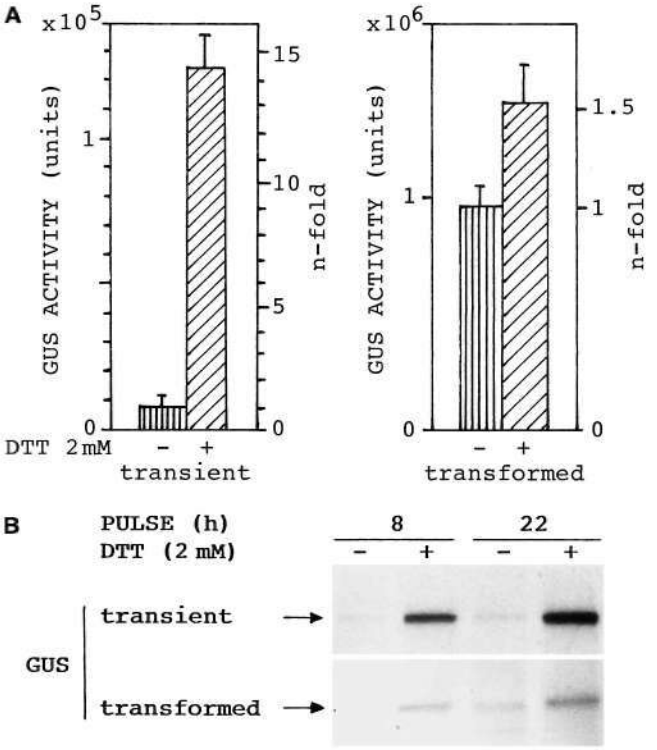

Fig. 1 A, B Effect of DTT on GUS activity and accumulation in protoplasts. **A** GUS activity (1 unit = 1 pmol of 4-methylumbelliferone/min/mg of protein; determined in 2.5×10^4 cells) in protoplasts cultured in the presence (+) or absence (–) of 2 mM DTT. Relative increases (*n-fold*) of activity due to DTT treatment are represented at the right of each graph. *Bars* represent the SE of the mean of three independent experiments. **B** Protoplasts expressing the 35S-*gus* gene construct under transient or permanent conditions were ^{35}S-labelled for the time periods indicated in the presence or absence of DTT, and GUS was immunoprecipitated and subjected to SDS-PAGE and autoradiography. Protoplasts were incubated in the dark

Results and discussion

The relative effect of DTT on GUS activity of protoplasts from tobacco plants transformed with a 35S-*gus* gene fusion was much smaller than that found in protoplasts transiently expressing the same gene construct (Fig. 1 A). To discern whether GUS protein present in protoplasts from transformed plants prior to DTT treatment was overshadowing the differential effect on its synthesis during the treatment period, GUS produced during this period was radioactively labelled, immunoprecipitated, and subjected to SDS-PAGE and autoradiography. As shown in Fig. 1 B, a clear increase in newly-formed GUS was observed in both cases. The increase in the two types of protoplasts was similar in relative terms; the weaker signal obtained for protoplasts from stably transformed plants reflected competition for the antibody by the excess non-labelled GUS present at the beginning of the experiment. This result indicated that the observed DTT effect was primarily due to the stress associated with protoplast isolation, although some contribution from the additional stress caused by electroporation could not be excluded (see Jones et al. 1987; Diaz 1994; and data in Fig. 5). In bacteria, GUS accumulates in the cytosol, where formation of disulfide bonds is prevented by the highly reductive environment that prevails in this compartment (Hwang et al. 1992). As has been previously reported for neomycin-phosphotransferase mRNA (Piñeiro et al. 1994), neither steady-state levels of GUS mRNA nor the efficiency of translation are affected by DTT, so it seems that oxidative stress resulting from the protoplast generation process itself must either cause the formation of spurious disulfide bonds in the nascent GUS, leading to the elimination of newly formed misfolded protein, or increase the protein degradation rate. In the first case, DTT would act by preventing GUS misfolding, whereas in the second case, its effect would be due to retardation of the degradation rate.

To investigate whether DTT treatment had any effect on degradation rate, GUS was radioactively labelled during transient expression in tobacco protoplasts cultured in the absence of DTT and then two aliquots of the labelled protoplasts were transferred into fresh medium with or without DTT. Degradation of labelled GUS was followed by immunoprecipitation, SDS-PAGE of the precipitate,

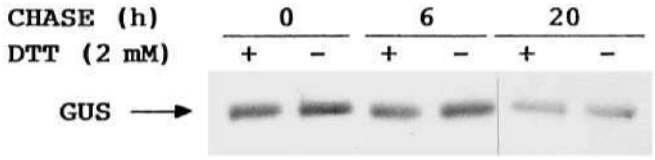

Fig. 2 Effect of DTT on GUS degradation. Protoplasts were radioactively labelled for 8 h and then cultured in fresh medium in the presence (+) or absence (–) of 2 mM DTT. GUS was immunoprecipitated and analyzed as in Fig. 1 B at the times indicated

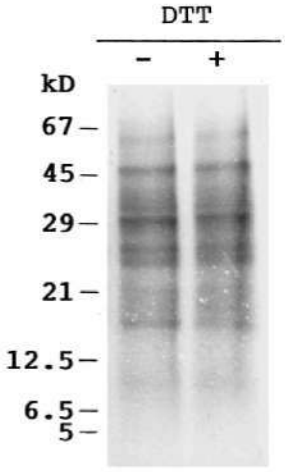

Fig. 3 Effect of DTT on synthesis of sulfur-containing cellular proteins. Protoplasts were labelled in the dark, in the presence (+) or absence (–) of 2 mM DTT, as in Fig. 1 B, and lysed in sample buffer after 22 h. Total proteins were subjected to SDS-PAGE and autoradiography

and autoradiography (Fig. 2). No effect of DTT on the degradation rate was observed, a result that supported the hypothesis that redox changes affect either a rate-limiting step in the folding of the nascent cysteine-rich protein and/or that the more oxidizing environment prevailing in the absence of DTT engenders more misfolded protein, which would be eventually degraded (Piñeiro et al. 1994).

In contrast to the effect on the reporter protein, DTT had no effect on the overall net accumulation of labelled plant cellular proteins, as judged from the electrophoretic patterns of total ^{35}S-labelled proteins separated by SDS-PAGE and observed by autoradiography (Fig. 3). This observastion did not exclude specific effects on proteins present at low concentrations, which would not be detected under the conditions used.

To investigate the possible effect of DTT on the protoplast protein export system, synthesis and export of PR proteins was studied, taking advantage of the fact that plant protoplasts spontaneously produce them as a result of the stress associated with the isolation procedure. Polyclonal antibodies that recognize chitinases and 1,3-β-glucanases were used to monitor their accumulation inside and outside the cell by Western blotting; no effect of DTT was observed (Fig. 4).

In these experiments, protoplast viability was >80% prior to electroporation and >50% after electroporation. This viability was stable during the incubation period and was not

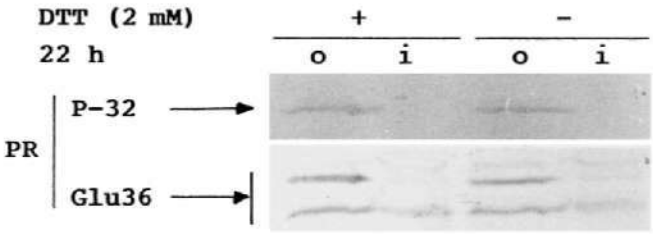

Fig. 4 Effect of DTT on synthesis and export of PR proteins. Western blot analysis of PR proteins P-32 and Glu-36 produced by protoplasts incubated in the dark in the presence (+) or absence (–) of 2 mM DTT. Both intracellular extract (*i*) and extracellular medium (*o*) were blotted. Medium was replaced before incubation

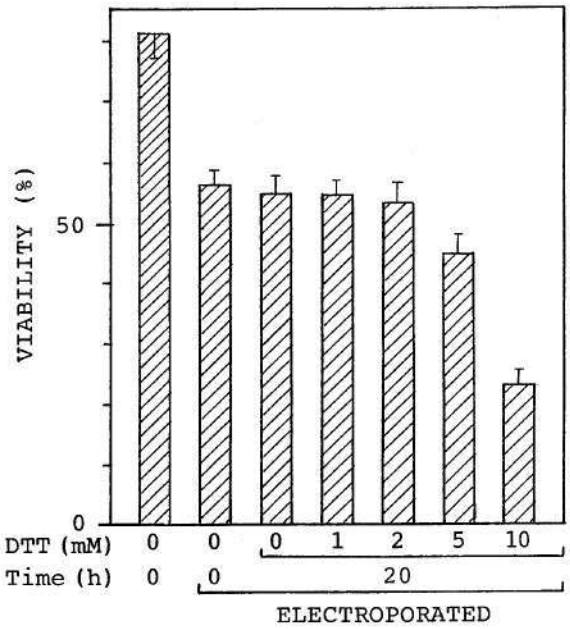

Fig. 5 Effect of DTT on viability of electroporated protoplasts. Viability was determined at the indicated times of culture, and expressed as the proportion (%) of protoplasts that excluded Evan's blue. Protoplasts were cultured in the dark. Maximal GUS activity (1.2×10^5 units) was reached at 2 mM DTT; activity at 5 mM and 10 mM DTT was >1% of the maximal activity. Bars represent the SE of the mean of four independent experiments

affected by treatment with DTT at the concentration used (Fig. 5). This was in line with the lack of effect of this reagent on the accumulation of total sulfur-containing cellular proteins or on synthesis and export of PR proteins.

In conclusion, the effect of DTT seemed to be rather specific for the reporter enzyme, which suggested that the redox cytoplasmic environment of the protoplast was unfavorable for an enzyme whose natural environment, the bacterial cytoplasm, is more reductive. This situation should be taken into consideration when using this enzyme as a transient-expression reporter under changing redox conditions.

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